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Original Article

Determining the clarithromycin-resistance of *Helicobacter pylori* in first-line therapy with melting curve analysis

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ABSTRACT

Aims: We aimed to determine the usefulness of melting curve analysis with the PCR technique in predicting the primary clarithromycin resistance of *H. pylori* before antibiotic use.

Methods: 46 *H. pylori* positive clarithromycin based therapy naïve patients were included in the study. After proving *H. pylori* positivity with a real-time test, histopathological examination and rapid urease test, patients were treated with clarithromycin-based triple therapy for 14 days. Then patients were underwent a control endoscopy and tissue samples were taken 8 weeks after the initial endoscopy. The presence of *H. pylori* was investigated by using TaqMan-based real-time PCR and clarithromycin resistance was determined using two different molecular methods:melting curve analysis and DNA sequencing.

Results: In total 18 patients (39.1%) were diagnosed as *H. pylori* positive on histopathological examination and 26 (56.5%) were diagnosed as *H. pylori* positive with TaqMan-based PCR, after triple therapy. According to results of melting curve analysis, primary resistance was found in 4 samples before treatment and secondary resistance was found in a different 4 samples after treatment. The DNA analysis showed that 9 patients had primary mutations in the pre-treatment group:4 patients had A2142C-A2143G(T), 3 patients had T2182C, and 2 patients had both mutations, and also showed that 6 patients had mutations in the post-treatment group:4 patients had A2142C-A2143G, 2 patients had T2182C mutations.

Conclusion: PCR based melting curve analysis may fail in determining clarithromycin resistance before eradication therapy. Hence, this method may also be used in combination with another molecular method such as DNA sequence analysis for improved detection of clarithromycin resistance.

Keywords: *Helicobacter pylori*, clarithromycin resistance, triple therapy, melting curve analysis

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is the most common gastrointestinal system related bacterial infection throughout the world, and has been suggested to cause gastritis, peptic ulcer disease, and gastric cancer. Clarithromycin based triple therapy is still the first line treatment for *H. pylori* infection, although reported evidence suggests high rates of resistance and, primary and acquired clarithromycin resistance is increasing worldwide.[1-3] Some common reasons for increased drug resistance are past medical history of previous clarithromycin usage and noncompliance to triple treatment. Although clarithromycin plays a key role in the treatment of *H. pylori*, increasing drug resistance has resulted in a failure of complete eradication of the bacteria.[4] Clarithromycin resistance has been reported to be 5%-10% in the USA, 1%-8% in Europe and changing heterogeneously from 11.4% to over 56% in Turkey according to results of previously published studies.[4-6]

In countries where the prevalence of clarithromycin resistance is high, determining the susceptibility of *H. pylori* against clarithromycin is so important before initiating an eradication regimen. Resistance to clarithromycin is a result of point mutations within the peptidyltransferase-encoding region of the 23S rRNA gene.[7] Three major point mutations in two positions on the 23S rRNA have been described: A2142C, A2142G, and A2143G.[7-10] A2142G and A2143G are the most frequently seen mutations, whereas mutation A2142C is less common. Point mutations at A2115G and G2141A have been shown to occur in the same strain.[9-11] Histopathology[12], *H. pylori* cell culture[13,14] and Fluorescence in Situ Hybridization (FISH)[15,16] are some of the current methods that are used for determining clarithromycin susceptibility. Several PCR-based techniques have been developed to determine these 23S rRNA related mutations.[11,17] A real-time PCR method which is based on amplification of a fragment of the 23S rRNA gene of *H. pylori* followed by melting curve analysis was also developed and used firstly by Gibson et al. on *H. pylori* strains.[18]

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In this study, we aimed to determine the usefulness of melting curve analysis with the PCR technique in predicting the primary clarithromycin resistance of *H. pylori* before antibiotic use in patients with confirmed *H. pylori* infection.

METHODS

Ethics

The study was carried out as thesis study in 2008. All ethical procedures and standards were carried out in accordance with the 1975 Helsinki Declaration.

Study Group and Sample Collection

The study was conducted at the Gulhane Military Medical Academy, Gastroenterology outpatient clinic, as a thesis study in year 2008, in Ankara, Turkey. A total of 46 *H. pylori* positive patients admitted to the gastroenterology outpatient clinic with chronic dyspeptic symptoms and who had not been treated with clarithromycin were included in the study. The presence of at least three of six symptoms (epigastric pain, heartburn, nausea, gas, bloating and belch) with a duration of not less than 4 weeks was established as the inclusion criteria for the study. Patients younger than 18 years or older than 65 years, those previously treated for *H. pylori* infection, those with a medical history of cholelithiasis, bleeding diasthesis, chronic disease or duodenum related surgery, drug history of non-steroidal anti-inflammatory drugs (NSAIDs) three months prior to endoscopy or erythromycin usage for any other reason were excluded from the study.

The gastric biopsy specimens were obtained from all patients during upper gastroduodenal endoscopy. A total of six biopsy specimens, including two specimens each from the antrum, and greater and smaller curvatures of the stomach, were obtained from all participants. For the Rapid Urease Test (Clo test), a biopsy specimen was obtained from the antrum and a specimen each from the antrum and greater curvature was obtained for the clarithromycin susceptibility studies. Patients with histopathologically confirmed diagnosis of *H. pylori* and a positive urease test were prescribed lansoprazole 30 mg capsule, amoxicillin 1000 mg tablet and clarithromycin 500 mg tablet all twice daily for 14 days. A control endoscopy was performed on all patients and biopsy samples were obtained again 8 weeks after the initial endoscopy.

Histopathological Examination

Biopsy specimens were put into sterilized containers, which included formaldehyde (10.0%), and transported to the pathology laboratory. All samples were embedded in paraffin. The paraffin-embedded tissue sections were stained using toluidine blue and hematoxylin and eosin, and examined with a light-microscope for the presence of *H. pylori* and other morphological findings.

DNA Extraction

For the clarithromycin susceptibility studies, collected biopsy specimens were stored at -20°C in Eppendorf tubes with 1ml 0.9% NaCl solution. Template DNA was extracted from biopsy specimens using the standard phenol-chloroform-isoamyl alcohol method.19 Briefly, approximately 40 mg of tissue samples were minced and suspended in 500 µl of TE buffer (10 mM Trishydrochloride, 1 mM EDTA, pH 8), and homogenized by vigorous mixing on a vortex. A 10 µl aliquot of protease solution (65 mg/ml) (Sigma-Aldrich Corp, St. Louis, MO, USA) and 250 µl of K buffer were added to the 250 µl of mixed specimen and incubated for 60 min at 45°C. Following centrifugation at 10,000 g for 10 min at 12°C, DNA was extracted from the supernatant using a mixture of 250 µl alkali phenol and 250 µl chloroform-isoamyl alcohol (24:1), and then precipitated using 500 µl isopropyl alcohol. The DNA was washed in 75% ethyl alcohol, centrifuged at 10,000 g for 5 min at 4°C, air-dried at 37°C and dissolved in 100 µl distilled water.

Real Time PCR Analyses

TaqMan based real-time PCR assays were used for the detection of *H. pylori* in biopsy specimens. The primers and probes used in this study are shown in **Table 1**. The reaction mixture was prepared for all real-time PCR assays as follows: 1.25 U Hot Start Taq DNA polymerase (Bioron, Germany), 10 pmol of each primer, 2.5 pmol TaqMan probe, 0.2 mM dNTP mix, and 2.5 mM $MgCl_2$. PCR amplifications were carried out in a final volume of 25 µL of the PCR reaction mixture, after the addition of 5 µl of the sample containing template DNA. The amplification conditions were as follows: Initial denaturation for 10 min at 95°C, followed by 40 amplification cycles of 15 s each at 95°C and 1 min at 60°C (annealing-extension step).[20] The primers and probes were designed using the OligoYap 4.0 software program.[21] All primer and probe sequences were analyzed with the GenBank BLAST database for specificity, and were synthesized by MWG-Biotech (Ebersberg, Germany). The TaqMan probes were labeled with a fluorescent reporter dye (FAM: 6-carboxy fluorescein) at the 5' end, and with black hole quencher (BHQ) as the non-fluorescent quencher at the 3' end. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control,[22] and the PCR mixture without the template DNA was used as a negative control. Bacterial isolates used as a positive control originated from the strains of our laboratory. The *H. pylori*-specific amplicons and the GAPDH amplicon were cloned into plasmid vectors using a TOPO TA Cloning System (Invitrogen, USA). Serial plasmid dilutions (108-101 copies/ml) were prepared for determining the detection sensitivities of the PCR assays. The calculation of standards was performed using spectrophotometry and OligoYap 4.0 software. All PCR analyses were performed using an iCycler thermal cycler instrument (Bio-Rad, USA).

Table 1. Primers and probes used in real-time PCR assays

Target genes		Primers and probes	Amplicon size
H. pylori	23S rRNA gene	F: 5'-agcattgtcctgcctgtggataa-3' R: 5'-ggttaaataccgacctgcatgaat-3' P (antisense): 5'-FAM-acctttactacaacttagcactgctaat-BHQ-3'	285 bp
Internal control (GAPDH)	Human GAPDH gene	P1: 5'-tcctgcaccaccaactgcttag-3' P2 : 5'- catcacrccacagyttyccagag-3' Probe: 5'-FAM-aggtcatccatgacaactttggyatcg-BHQ -3'	145 bp

Abbreviations: F: forward primer, R: reverse primer, and P: probe.*R and Y codes indicate degenerate bases; R=(A/G), and Y=(C/T). bp (base pairs).

Melting Curve Analysis

SYBR Green-based PCR reactions were performed for melting curve analysis using the same primers that were used in the TaqMan-based type-specific real-time PCR (**Table 1**). PCR reactions were carried out in a total of 25 µl volumes containing 10 pmol of each primer, a final concentration of 1X SYBR Green I, 0.25 mM dNTPs, 2.0 mM $MgCl_2$, and template DNA. PCR amplification cycles were as follows: a single cycle for 10 min at 95°C (hot-start Taq DNA polymerase activation), followed by 40 amplification cycles of 15 sec each at 95°C and 1 min at 60°C (annealing-extension step). After completion of the 40 PCR cycles, the melting-curve data were obtained by continuous fluorescence acquisition from 55 to 95°C, with a thermal transition rate of 0.1 °C/s.[22]

DNA Sequencing

PCR amplicons were obtained in our laboratory by using the sense and antisense primers from biopsy specimens that revealed the presence of bacteria before and after treatment, and sent to the MWS Company (Germany) for DNA sequencing. A total of 58 *H. pylori* positive samples (39 obtained before treatment and 19 samples obtained from the post-treatment group) were sequenced, while other samples were evaluated that were not suitable for sequence analysis.

Statistical Analysis

The Chi-square test was used to evaluate the statistical significance of the difference between groups, and p values <0.05 were considered significant (at the 95% confidence interval). Statistical analyses were conducted using the Statistical Package for the Social Sciences (SSPS) 15.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Of the 46 patients, 14 (30.4%) were males and 32 (69.6%) were females. The mean age of the patients was 37.05 ±11.57. When pre-treatment and post-treatment findings were compared among patients, the relationships among response rate, age, gender, endoscopic findings, treatment response, smoking and mutant genes did not show any significant differences.

Histopathological findings revealed that *H. pylori* positivity was present in 46 (100%) patients before treatment and in 18 (39.1%) patients after treatment. The PCR technique detected *H. pylori* positivity in 46 (100%) patients before treatment and in 26 (56.5%) patients after treatment. By using the TaqMan PCR, the analysis we conducted showed positivity for *H. pylori* in all of the 46 collected biopsy specimens before treatment and positivity in 26 biopsy specimens after treatment. As a result of our SYBR Green I and Melting Curve Analysis study, we detected primary resistance in 4 biopsy samples collected before treatment and secondary resistance in a different 4 biopsy specimens after treatment.

DNA sequencing analysis of *H. pylori* strains was successfully accomplished in 39 patients before treatment and 19 patients after treatment. All other samples were not found to be suitable for sequencing analysis. DNA sequence analysis detected resistant strains in 15 patients (34.8%) from both groups: 9 of these patients had primary mutations in the pre-treatment group, and 6 patients had mutations in the post-treatment group. The mutations found in the post-treatment group were not part of the pre-treatment primary resistance but rather developed during treatment. Results from DNA sequencing analysis in both pre treatment and post treatment groups are shown in **Table 2**.

Table 2. Patients have clarithromycin resistant *H. pylori* strains before and after treatment

	Mutation types	Number of patients
Pre treatment (39 patients)	A2142C-2143G(T)	4 (10.26%)
	T2182C	3 (7.69%)
	Concurrent (double) mutations; A2142-C2143G(T) and T2182C.	2 (5.13%)
	Total	9 (23.08%)
Post treatment (19 patients)	A2142C-2143G(T)	4 (% 21.05)
	T2182C	2 (10.52%)
	Concurrent (double) mutations	0
	Total	6 (%31.57)

Graphics of the Melting Curve analysis are shown in **Figure 1**, **Figure 2** and **Figure 3**.

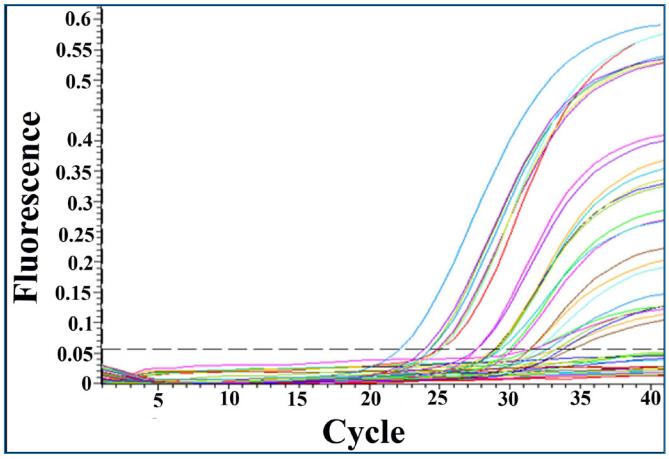

Figure 1. TaqMAN real time PCR peaks of *H. pylori* positive and negative samples. The peaks under dotted line are negative peaks

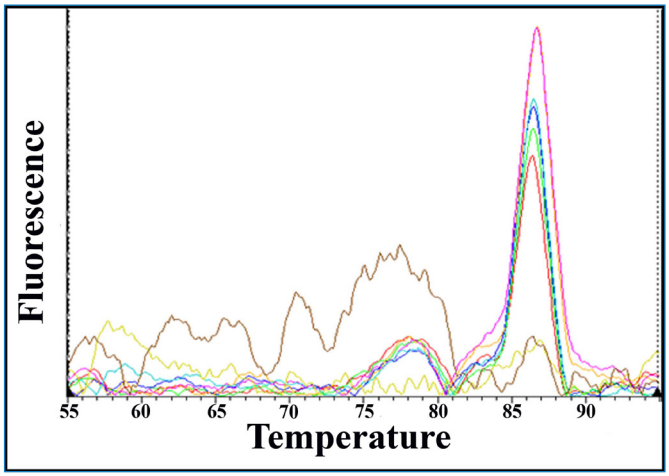

Figure 2. Melting curve graphic of *H. pylori* positive and irresistant samples between 86.2–86.9°C

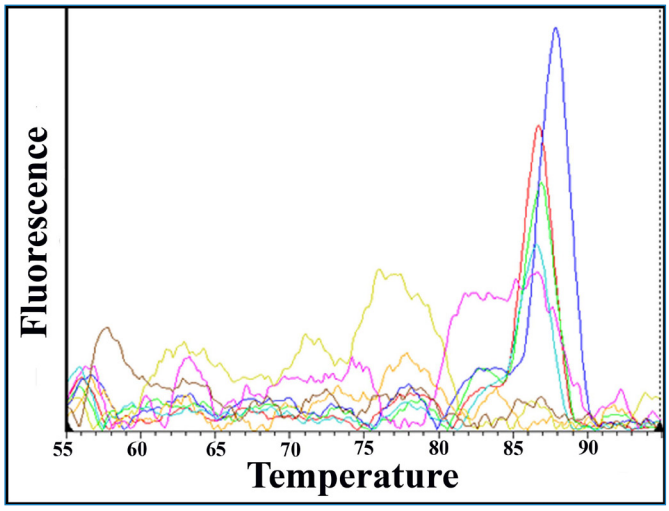

Figure 3. Melting curve graphic of *H. pylori* positive and resistant/ irresistant samples between 86.5–87.1°C. Blue peak is at 87.1°C and different from normal appearing.

DISCUSSION

Clarithromycin resistance to *H. pylori* occurs as a result of single-base mutations within the peptidyltransferase-encoding region of the 23S rRNA gene. The most commonly encountered mutations are A2142G, A2143G, A2144G, C2182T, and with a lower incidence A2143C. Other rarely seen mutations are G2141A, A2143T, T2183C, T2245C, A2144T and T2717C. Distribution of these mutations show regional differences.[7-11]

In previous research, Chen et al. demonstrated A2143G mutation in 11.11% and C2182T mutation in 12.96 % of 54 patients before treatment, via DNA sequencing using the PCR technique. This was later confirmed by DNA sequencing analysis.[23] In Chile, Garrido et al.[24] studied 50 patients with no history of clarithromycin exposure and found primary resistance in 10 (20%) of the patients, using the agar dilution method. Point mutations were observed in four of the ten patients and two or more mutations were observed in other six cases. In 9 patients, A2142G mutation was seen and A2143G mutation was observed in only one patient. Other detected mutations from the isolates were C2147G, G1939A, T1942C and A2142G. Similar mutations were seen after nucleotide sequencing. In our study, out of the 39 patients who were considered appropriate for DNA analysis, we found primary resistance in 4 (10.26%) patients with A2142C-2143G(T) mutations, in 3 (7.69%) patients with T2182C mutation, and in 2 (5.13%) patients with both mutations. We found the total mutant gene ratio to be 23.08%. This result was similar to the resistance ratio (20%) of Garrido et al.'s study.[24] Of the 9 patients, in whom primary resistance was detected prior to treatment, complete eradication of *H. pylori* failed in 3 patients and the remaining 6 patients exhibited absence of *H. pylori* after treatment. Among the 19 patients, in whom DNA sequencing analysis was performed after treatment, we found A2142-2143G mutations in 21.05% (4/19) of patients and T2182C mutation in 10.52% (2/19) of patients. These mutations were not a part of pre-treatment primary resistance, but rather developed during treatment. Following treatment, A2142-2143G and T2182C point mutations were seen to be absent in resistant strains. The mutant gene ratio found in our study is closer that in the results from Chen et al.'s[23] study, which was conducted in China.

In another *H. pylori* study, 470 patients were enrolled by Mahachai et al.[25] the eradication rate was 90.6% in clarithromycin sensitive patients compared to an eradication rate of 56.3% in clarithromycin resistant patients. Also in a similar study conducted by Vincenzo et al.[26] 75 patients with *H. pylori* were given PPI, amoxicillin and clarithromycin for seven days. Upon further evaluation, eradication was achieved in 11(48%) of the 23 patients who showed A2143G mutation-associated clarithromycin resistance and 14 (93%) patients with resistance due to either A2142G or A2142C mutations.

Of the six patients in whom we detected secondary resistance, histopathological findings revealed failure of *H. pylori* eradication in four patients. However, two patients were completely eradicated. Among the patients with secondary resistance, we found an eradication rate of 33.34%. In the paper by Chen et al.[27] while *H. pylori* positivity was found in all patients via PCR, histopathological evaluation revealed two-thirds of the patients to be positive. The difference in results was attributed to the change in position of specimens during the study. In our study, while histopathological findings following treatment revealed *H. pylori* positivity in 18 (39.1%) patients, control PCR testing showed the presence of *H. pylori* in 26 (56.5%) patients. Also with DNA sequencing analysis, we found point mutations in *H. pylori* that led to clarithromycin resistance in six of these patients. According to the results of melting curve analysis, two of the four patients who showed resistance to clarithromycin after PCR prior to treatment were found to have *H. pylori* positivity after treatment. This finding was confirmed by histopathology: positive *H. pylori* results were observed from both histopathological evaluation and PCR analysis conducted in all four patients post treatment. After DNA sequencing analysis studies, histopathology results revealed *H. pylori* positivity in 3 of the 9 patients in whom mutant genes were detected before treatment, whereas 4 of the 6 patients who had mutant genes after treatment still showed *H. pylori* positivity.

A close look at the results of existing studies reveals that, irrespective of the method used in determining clarithromycin resistance or sensitivity, in patients who have tested positive for *H. pylori* undergoing first line treatment with clarithromycin based triple therapy, complete eradication can either be achieved in resistant cases or fail in sensitive cases.[25-29] This finding clearly shows that current methods used in determining clarithromycin susceptibility posses existing flaws that need to be addressed.

CONCLUSION

The aim of determining clarithromycin resistance before treatment we were able to show that, results from both the PCR and Melting Curve Analysis method may not be solely true or reliable. From this perspective, we inferred that in determining clarithromycin resistance via molecular methods, instead of using the Melting Curve Analysis method only, combining it with other methods such as DNA sequencing analysis may be useful. In the light of the current study, we are of the view that, further development and improvements in the Melting Curve Analysis Method for determining clarithromycin resistance in microbiology laboratories could be useful, as well as cost and time effective, in clinical practice and as such play a larger role in patient care.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out as thesis study in 1999.

Informed Consent: Due to the nature of the study, informed consent is not required.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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